ARTICLE

Protocol for Optimal Quality and Quantity Pollen DNA Isolation from Honey Samples

Ralte Lalhmangaihi,[1] Souvik Ghatak,[2] Ramachandra Laha,[1] Guruswami Gurusubramanian,[3] and Nachimuthu Senthil Kumar[2]

Departments of [1]Botany, [2]Biotechnology, and [3]Zoology, Mizoram University, Aizawl, Mizoram, India

The present study illustrates an optimized sample preparation method for an efficient DNA isolation from low quantities of honey samples. A conventional PCR-based method was validated, which potentially enables characterization of plant species from as low as 3 ml bee-honey samples. In the present study, an anionic detergent was used to lyse the hard outer pollen shell, and DTT was used for isolation of thiolated DNA, as it might facilitate protein digestion and assists in releasing the DNA into solution, as well as reduce cross-links between DNA and other biomolecules. Optimization of both the quantity of honey sample and time duration for DNA isolation was done during development of this method. With the use of this method, chloroplast DNA was successfully PCR amplified and sequenced from honey DNA samples.

KEY WORDS: bees, chloroplast DNA, polliniferous plants

INTRODUCTION

Honeybees have been described as the most useful of all insects known to mankind.[1] Honeybees achieve ~80% concentration of sugars in honey[2] by adding enzymes that convert the sugars into more water-soluble kinds and thereby, allowing for large amounts of water evaporation. The high sugar content, combined with other enzymes added by the honeybees, makes them able to possess antimicrobial properties and makes long-term storage possible and decomposition less likely.[3] The spectrum of pollen varieties indicates the plants visited by bees during the production of the honey and permits the characterization of the geographic origin.[4]

Plant identification is challenging when no morphological assignable parts are available. Other than identifying whole plants, it is also sometimes useful to be able to identify species from material, such as roots, seeds, or pollen or in mixtures of plants sampled from the air, soil, or water, although this may be difficult or impossible using traditional morphological approaches.[5] The fact that DNA from the plant(s) may be present in honey represents a useful, analytical tool to identify the host plants.[6] The haploid microspores of seed plants (the pollen) are the male part in sexual reproduction of flowers. Pollen grains have a very hard outer shell, called the exine, which is tough and can be found in fossil deposits, millions of years old. For DNA extraction from pollen grains, it is necessary to find an effective method to destroy the exine.

DNA-based analytical methods are less dependent on the analyst and can be applied in different laboratories equipped with suitable instruments. It is possible to identify the plant species as well as microflora of honey from the single isolated honey DNA. Honey rarely comes from a single plant species, even if it is attributed to a single species. Taking into account the health risk from plants producing toxins, it is crucial to assess correctly the identity of the plants from which pollen comes. Traditionally, the determination of the floral composition of honey has been achieved by the melissopalynology method, which is based on the identification of pollen by light microscopy.[4] However, it requires highly specialized researchers, and for this reason, there is the need for an alternative and sensitive method. From this point of view, the application of molecular methods to the floral analysis in honey offers the possibility to detect a much greater range of plant species in honey, overcoming the limitations of the morphological identification of plant pollen and spores.

The present study aims to develop a simple and efficient method for the extraction of PCR amplifiable chloroplast DNA (cpDNA) with a reduced sample amount of 3 ml honey compared with previous studies (e.g., 10 ml by Cheng et al.[6] and 10 g by Laube et al.[7]). As other honey DNA isolation methods are mostly described as kit-based approaches, we developed an easy and efficient method for

Address correspondence to: Ramachandra Laha, Dept. of Botany, Mizoram University, Aizawl 796 004, Mizoram, India (Phone: +91-9862769353; E-mail: rc_laha@yahoo.com).
doi: 10.7171/jbt.14-2504-001

honey DNA isolation by conventional phenol-chloroform methods. We additionally show that the extracted DNA is of sufficient quality and quantity to enable PCR amplification and sequencing of genetic markers routinely used for plant barcoding. Accordingly, the DNA can be used to investigate the plant source of origin of the honey samples.

MATERIALS AND METHODS

Sample Collection and Study Sites

Eight honey samples were collected from different hives in Aizawl, Mizoram, India. The hives were inhabited by *Apis cerana*, and all of the samples were located near the beekeeper house, where much of the nectar collected by the bees was likely from the farmland or the garden of the residential properties in the area. The honey samples were stored at 4°C before DNA isolation.

DNA Isolation from Honey Samples

Honey (3 ml; which might contain pollen cells derived from the plant DNA) was dissolved in 1 ml sterile water and incubated at 65°C for 30 min, followed by centrifugation at 5000 rpm for 10 min. The supernatant was discarded, and the pellet was dried for 5 min at room temperature and dissolved in 500 μl extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 50 mM NaCl, 10% SDS, pH 7.5). Sterilized glass beads 0.5 g (diameter 0.5–1 mm) were added, and the pellet was ground with a glass rod for 5–10 min. DTT (100 μl; 110 mM) and 10 μl proteinase K (10 mg/ml) were added, mixed by gentle inversion, and incubated at 56°C for 1 h, followed by addition of 500 μl cetyltrimethyl ammonium bromide (CTAB) extraction buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 10% CTAB, 5% polyvinylpyrrolidone), 10 μl proteinase K, and 50 μl DTT and incubated at 65°C overnight in water bath. Phenol-chloroform-isoamyl alcohol (500 μl) was added and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a 2 ml Eppendorf tube, followed by adding 500 μl isopropanol and 100 μl sodium acetate (3 mM) and kept in −20°C for 1 h for precipitation. The sample was centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was mixed with 400 μl 70% ethanol and subsequently incubated at −20°C for 15 min. The sample was centrifuged at 13,000 rpm for 10 min at 4°C, the supernatant was poured off, and the pellet was dried in oven. Millipore water (30 μl) was added to the tube and mixed gently. Yield and DNA purity were checked by using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The quality of the extracted DNA was checked by using a 0.8% agarose gel in 1× Tris-acetic acid-EDTA buffer at 80 V for 30 min, stained with ethidium bromide. Images were obtained in a G:BOX gel documentation system (Syngene, Cambridge, UK).

PCR Amplification of cpDNA

PCR was performed with a set of primers amplifying a fragment of the plant cpDNA maturase K (*mat*K) universal barcoding region[8] (Table 1). The PCR products (10 μl) were subjected to electrophoresis using a 1.2% agarose gel, following the same conditions as above. PCR products were purified using a Qiagen gel extraction kit (QIAquick columns; Qiagen, Chatsworth, CA, USA) and stored at −20°C until sent for sequencing to SciGenom Labs (Cochin, India).

Sequence Analysis

The sequence file was annotated based on the chromatogram and BLAST results. Reference and query sequence alignments were simulated using a readily available pipeline in the National Center for Biotechnology Information (NCBI) BLAST program. Genetic distance was used to assign an identity to each query sequence, and the ID of the plant species was associated with the best BLAST hit and E-value < cutoff. This corresponds to choosing the top hit in the BLAST results. The value range will depend on the query and identity coverage.

A local installation of BLAST was used to search for the query sequence among the reference sequences. In each

TABLE 1

List of Primers and Their Conditions for Amplification of *mat*K Genes

Primer name	Primer sequences (5′–3′)	PCR mix	PCR conditions	Expected products length, bp
*mat*K22F	CGATCTATTCATTCAATATTC	100 ng–template DNA	94°C–40 s	
		1.6 pM–each primer	49.5°C–40 s	900
*mat*K22R	TCTAGCACACGAAAGTCGAAGT	1×–PCR buffer	72°C–60 s	
		1.5 mM–$MgCl_2$	(35 cycles)	
		0.25 M–dNTPs		
		1 U–Taq polymerase		

The PCR consisted of 25 μl total reaction volume, and condition for the genes involves an initial denaturation at 95°C for 5 min and a final extension at 72°C for 10 min.

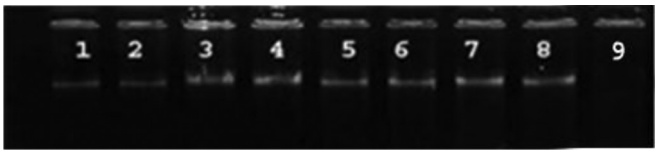

FIGURE 1

DNA samples extracted from honey samples. Sample Numbers 1–8, individual samples; 9, negative control.

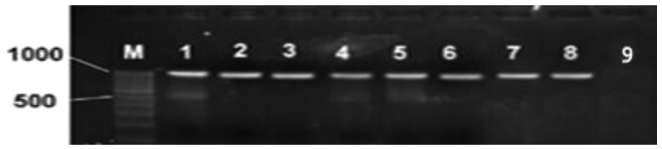

FIGURE 2

PCR-amplified products of the cpDNA matK (900 bp) region from honey DNA. M, 100-bp marker; Sample Numbers 1–8, PCR-amplified, individual samples; 9, negative control.

case, a liberal (10–2) or more restrictive (10–6) E-value cutoff was used. The query's ID was uncertain when no hits had an E-value below this cutoff.[9]

RESULTS

There is a critical factor that needs scrupulous attention when a PCR-based method is applied to the analysis of honey DNA samples: honey consists of at least 80% sugar, and this may act as an inhibitory factor for the PCR. As a consequence, the DNA extraction protocol has to be optimized to ensure a sufficient amount of DNA, free of PCR-inhibiting substances. We found that a preliminary, extensive 65°C incubation (for 1 h) and glass-beads grinding of the samples were very important to minimize the effect of high concentration of polysaccharides, and the lysis of the pollen exine was done in an Eppendorf tube. As a pilot study, the amount and purity of DNA extracted from the sample were determined using spectrophotometry, and the total DNA yields of each honey sample ranged from 20 to 45 ng/μl (Fig. 1 and Table 2). However, the 260/280 optical densities (ODs), a measure of extract purity, varied from 1.65 to 1.82 (Table 2). As shown in Fig. 1, the extracted DNA was intact, the method provided positive results in all of the genomic DNA samples, and it shows a high molecular-weight, PCR-amplified band (900 bp) in the gel (Fig. 2).

DNA sequences were subjected to the BLAST program in NCBI. Number 1 sample matches 100% with species *Tetrameles nudiflora* within the family Datiscaceae; Number 4 sample with species *Thouinidium decandrum, Dimocarpus longan*, and *Litchi chinensis* from the Sapindaceae family; and Number 6 sample with species *Mikania guaco* and *Dyscritothamnus mirandae* within the Asteraceae family. The BLAST method, which is to accept the top hit as the species identification, performed the best, and all subsequent discussion of BLAST methods in the present work will refer only to this method.

TABLE 2

Quantity and Quality of the DNA Extracted from Honey Samples

Sample number	DNA yield, ng/μl	DNA purity, A_{260}/A_{280}
1	20	1.82
2	23	1.79
3	43	1.67
4	45	1.62
5	27	1.79
6	29	1.81
7	35	1.72
8	36	1.68

A_{260}/A_{280}, Ratio of absorbance at 260/280 nm; sample numbers 1–8, individual honey samples.

DISCUSSION

The results demonstrate that the DNA isolation method is successful, even in farm samples, where the sugar content will be high. DTT was used as a reducing agent for thiolated DNA. The terminal surfur atoms of thiolated DNA have a tendency to form dimers in solution, especially in the presence of oxygen,[10] and honey contains lots of different biomolecules, which form cross-link with the DNA. DTT was used for isolation of thiolated DNA, as it would facilitate protein digestion and assist in releasing DNA into the solution. The most important part of the DNA isolation method is the time duration involved in grinding sample by glass beads, which determines the pellet formation after centrifugation. The excess sugar content in honey samples is also a main problem for good DNA yield. In the present study, three samples had a OD value of 1.6, which indicates the presence of saccharides. Hence, removal of sugar from the honey samples will be important during the DNA extraction process. On the other hand, the high carbohydrate concentration in the honey helps DNA preservation,[11] as sugars stabilize nucleic acids,[12] and honey provides an airtight seal that prevents oxygen from entering and thus, preserving DNA from being degraded.[11] The presence of polyphenolic content makes the isolation of high-quality nucleic acids problematic; in addition, residual polyphenolics interfere in enzymatic reactions, such as PCR and endonuclease restriction digestion.[13] In the present study, initial incubation of the sample dissolved in water resulted in removal of sugar and polyphenols as supernatants after the centrifugation process.

In the present study, the choloroplast *mat*K gene was amplified successfully from the DNA isolated from honey samples. It is difficult to amplify the cpDNA of different plants from a single honey samples, as PCR primers need perfect conditions to track the exact position for amplification of degraded DNA,[14] as remains in decomposing plants.[15] The informal identification of an unknown plant specimen from honey samples using BLAST, to search large public databases, may be as reliable a method as any other. Furthermore, the application of a decision criterion that sums the "weight of evidence," which integrates across top-ranking BLAST hits, is no more reliable than simply using the best hit.[9] However, the reliability of BLAST is mainly dependent on the comprehensiveness of the taxon representation in the database. The altering of the E-value cutoff to more or less restrictive values will tune down or up the probability of BLAST incorrectly making a positive identification. The E-value—the probability of a random match having the observed quality—is proportional to the size of the sequence search space so that increasing either the number or length of sequences in the reference database will reduce the E-value of a given match.

It is noteworthy that Datiscaceae, Sapindaceae, Asteraceae families are important in the bee foraging, as they include many nectariferous and polliniferous species. The identification of these families reflects the abundance of the flora surrounding the sample collection site. We found that high DNA content of honey samples is typical of unifloral *D. longan* and *L. chinensis* species honey and high pollen content, confirmed by the dark amber color, rather than any other planlonganles.[11,16]

Conclusion

The methodology used in the present study is indigenous, cost effective, and comparatively less time consuming than any other conventional methods. The present method is also advantageous, as high DNA harvest was obtained with low quantity of the honey sample. The DNA extraction method using DTT, high salt, and the anionic detergent solution developed in this study represents a simple protocol that excels the cpDNA amplification success rate by the kit method for honey DNA and subsequent PCR amplification. Although detailed investigation of how the content varies with regard to both the stated plant sources—geographic regions and farming type—would be of interest.

ACKNOWLEDGMENTS

This work was supported by the State Biotech Hub and Bioinformatics Infrastructure Facility, Mizoram University, sponsored by the Department of Biotechnology (DBT; New Delhi, India).

REFERENCES

1. Adjare SO. *Beekeeping in Africa*, Bulletin 68/6. Rome: Food and Agriculture Organisation of the United Nations, 1990.
2. Chemas A, Rico GV. Apiculture and management of associated vegetation by the maya of Tixcacaltuyub, Yucatan, Mexico. *Agrofo Syst* 1991;13:13–25.
3. Crane E. *The World History of Beekeeping and Honey Hunting*. Oxon, UK: Routledge, Taylor and Francis group, 1999.
4. Louveaux J, Maurizio A, Vorwohl G. Methods of melissopalynology. *Bee World* 1978;59:139–157.
5. CBOL Plant Working Group. A DNA barcode for land plants. *Proc Natl Acad Sci USA* 2009;106:12794–12797.
6. Cheng H, Jin W, Wu H, et al. Isolation and PCR detection of foreign DNA sequences in bee honey raised on genetically modified Bt cotton. *Food Bioproc Proc* 2007;85:141–145.
7. Laube I, Hird H, Brodmann P, et al. Development of primer and probe sets for the detection of plant species in honey. *Food Chem* 2010;118:979–986.
8. Yu J, Xue JH, Zhou SL. New universal *mat*K primers for DNA barcoding angiosperms. *J Syst Evol* 2011;49:176–181.
9. Ross HA, Murugan S, Li WL. Testing the reliability of genetic methods of species identification via simulation. *Syst Biol* 2008; 57:216–230.
10. Lexa GM, Francis NW, Miriam GK, Anne WT, Titus KM. Leaf storage conditions and genomic DNA isolation efficiency in *Ocimum gratissimum* L. from Kenya. *Afri J Biotech* 2008;7:557–564.
11. Valentini A, Miquel C, Taberlet P. DNA barcoding for honey biodiversity. *Diversity* 2010;2:610–617.
12. Schill RO, Mali B, Dandekar T, Schnölzer M, Reuter D, Frohme M. Molecular mechanisms of tolerance in tardigrades: new perspectives for preservation and stabilization of biological material. *Biotechnol Adv* 2009;27:348–352.
13. Michiels A, Vanden-Ende W, Tucker M, Van-Riet L, Van-Laere A. Extraction of high-quality genomic DNA from latex containing plants. *Anal Biochem* 2003;315:85–89.
14. Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C. Power and limitations of the chloroplast *trnL* (UAA) intron for plant DNA barcoding. *Nucl Acid Res* 2007;35:14–21.
15. King R, Read D, Traugott M, Symondson W. Molecular analysis of predation: a review of best practice for DNA-based approaches. *Mol Ecol* 2008;17:947–963.
16. Persano-Oddo L, Piazza MG, Sabatini AG, Accorti M. Characterization of unifloral honeys. *Apidologie* 1995;26:453–465.